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An attenuated West Nile prototype virus is highly immunogenic and protects against the deadly NY99 strain: a candidate for live WN vaccine development

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Abstract

In a short time, West Nile virus has developed into a nationwide health and veterinary problem. The high virulence of the circulating virus and related lineage 1 WN strains hinders development of an attenuated live vaccine. We describe an attenuated WN isolate, WN1415, which is a molecularly cloned descendant of the WN prototype B956 strain. The parent virus belongs to lineage 2, members of which have not been associated with epidemic or epizootic outbreaks. A set of non-conservative mutations, mostly in non-structural protein genes, distinguishes the WN1415 isolate from the parent B956 prototype strain. Immunization with WN1415 (55–550,000 pfu) established a potent immunity, which protected the majority of mice against lethal challenge with WN NY99. The attenuated nature of the isolate and its excellent growth characteristics combined with the availability of a highly stable infectious clone make the isolate an attractive candidate for live WN vaccine development.

Keywords: Flavivirus; West Nile virus; B956 strain; NY99 strain; Attenuation; Vaccine; Immunization; Immune response; Antibody; Neutralization; Mouse

Introduction

West Nile virus was first isolated over 60 years ago from the blood of a febrile patient (Smithburn et al., 1940), and it is one of the most widespread flaviviruses worldwide. The virus is endemic to Africa and has been repeatedly registered in Europe and Asia for decades causing self-limiting epidemics and epizootics (Murgue et al., 2001; Savage et al., 1999). Recent introduction of the virus into the naïve environment of the North American continent (Lanciotti et al., 1999) had disastrous consequences both for wildlife and human population (Roehrig et al., 2002) and in a few years has developed into a nationwide epidemiological problem. In addition to hundreds of human mortality cases reported to the CDC (CDC, 2004), the virus imposes a substantial economical burden, especially on the equine industry (Anonymous, 2003).

A veterinary vaccine of moderate efficacy based on a formalin-inactivated circulating strain has been developed for equine immunization (Kahler, 2003; Ng et al., 2003; Nusbaum et al., 2003). Experimental chimeric vaccines based on yellow fever 17D and dengue virus vectors have entered clinical trials (Lai and Monath, 2003). Viable chimeric flaviviruses carrying WN structural protein genes induce high titers of virus-specific neutralizing antibodies and protect mammals against challenge with pathogenic WN virus (Pletnev et al., 2002; Tesh et al., 2002). YF-based WN vaccine was not effective in avian species, which is a consequence of the host restriction imposed by the vector (Langevin et al., 2003). Cell-mediated immune response plays an important role in virus clearance and in protection

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from the disease (Diamond et al., 2003; Shrestha and Diamond, 2004). Since flavivirus nonstructural proteins supply the majority of dominant T-cell peptide determinants (Co et al., 2002), cell-mediated response induced by chimeric flaviviruses would be mostly to vector proteins. However, development of a live attenuated WN vaccine, which may have a better capability to elicit balanced humoral and cell-mediated immune responses, is hindered by the high virulence and pathogenicity of the NY99 strain circulating in the U.S. (Beasley et al., 2002; Roehrig et al., 2002).

Based on serological data and genetic characterization, West Nile viruses were subdivided into two distinct lineages (Berthet et al., 1997; Price and O'Leary, 1967). Viruses of lineage 1, which includes the highly virulent NY99 strain, are most widespread and often were found in association with epidemics or epizootics (Roehrig et al., 2002). Although a few strains with a high virulence were also found among lineage 2 representatives (Beasley et al., 2002), viruses of this lineage have not been associated with disease outbreaks (Lanciotti et al., 1999). For this reason, lineage 2 viruses may be more attractive for development of live attenuated West Nile vaccine. We report here the identification of a potential candidate for development of such vaccine, which is a descendant of the West Nile virus prototype B956 (Smithburn et al., 1940) and is one of the first flaviviruses for which the complete nucleotide sequence has been determined (Castle et al., 1985, 1986; Wengler et al., 1985). Earlier, we have reported construction of the first WN infectious clone designed on the basis of the isolate 956D117B3, a laboratory derivative of B956 (Yamshchikov et al., 2001). In this report, we demonstrate that virus recovered from the molecular clone is highly attenuated, induces vigorous and balanced immune response and even at low doses protects mice against the virulent NY99 strain. Combined with its stable genotype and excellent growth characteristics in tissue culture, the recovered virus could be used in development of veterinary and human live West Nile vaccines.

Results

Isolation of WN 1415, a molecular clone of the 956D117B3 virus

Isolate 956D117B3 (Wengler and Beato, 1979; Yamshchikov et al., 2001) is a descendant of WN prototype strain B956 (Smithburn et al., 1940), which since its original isolation underwent numerous laboratory passages. Subcloned cDNA of this isolate was used to determine the complete nucleotide sequence of the WN virus genome (Castle and Wengler, 1987; Castle et al., 1985, 1986; Wengler et al., 1985). In our previous work, the 956D117B3 isolate, which was considered lost, was rescued from archived RNA and at Vero passage 2 was used to design the first infectious clone of WN virus (Yamshchikov et al., 2001). Plasmid pWN1415, a molecular clone, was used to prepare synthetic RNA, transfection of which into BHK cells gave rise to a virus designated WN1415. For genetic characterization, total RNA was isolated from Vero cells infected with WN1415 at passage 2 after transfection. Direct sequencing of gel-purified cDNA fragments prepared from this RNA has revealed two silent substitutions in positions 4566 (C) and 8355 (U) of the WN1415 genome as compared to the reported nucleotide sequence of 956D117B3 (GenBank #M12294); no other differences were identified. The above substitutions, which are present in the infectious clone plasmid pWN1415 as well, likely constitute cDNA variants selected during cloning. We have verified the nucleotide sequence of the parent 956D117B3 isolate at Vero passage 2 after its rescue from archival RNA. Consensus sequencing performed without intermediate subcloning of cDNA produced a sequence identical to that reported earlier (GenBank #M12294), but also revealed several heterogeneous loci containing minor peaks, which indicated the presence of quasi-species in the viral population. In contrast, these heterogeneous loci were not found in the consensus sequence of the WN1415 clonal isolate characterized at the same passage level after recovery from RNA, thus indicating a homogeneous viral population.

Sequence analysis of parent B956 and its descendants

To determine the extent of changes accumulated in the 956B117D3 genome over its passage history, we have obtained the original B956 strain at mouse brain passage 2. The virus was passaged once in C6/36 cells and total RNA isolated from virus-infected cells was used for preparation of purified cDNA fragments. The nucleotide sequence of the entire B956 genome was determined without intermediate subcloning of cDNA fragments. Comparison of the B956 sequence with the reported sequence #M12294 of the 956D117B3 isolate (earlier also referred to as WN-Nigeria or WN-Wengler (Berthet et al., 1997; Lanciotti et al., 1999)) revealed 32 point mutations, resulting in 14 amino acid changes spread over the entire genome (Table 1). Most of these amino acid changes accumulated in NS4A, followed by NS4B, with only 3 found in the structural protein region, suggesting that most of the changes are associated with intracellular replication, presumably with a little or no effect on the virion properties. The most prominent is a 76 nt deletion in the 3′-UTR beginning after pos. 10391 in the B956 genome, that is, immediately downstream from the stop codon terminating the WN open reading frame. Two nucleotide changes were also found in the 3′-terminal stem-and-loop structure, which is conserved in flaviviruses and is important for RNA replication (Proutski et al., 1999). When the 3′-terminal 110 nucleotides of either sequence were folded using MFOLD (Zuker, 2003), both these changes were found in unpaired bulges of the otherwise completely conserved structure (results not shown) and thus probably do not play important roles.

Table 1
Summary of sequence differences between B956 and 956D117B3

Position[a]	Gene	B956p2	956D117B3	Change
65	5′-UTR	**U**	**C**	–
327	C	GC**G**	GC**A**	–
540	prM	AU**C**	AU**A**	–
975	E	UG**C**	UG**U**	–
1063	E	**C**UA	**A**UA	Leu→Ile
1548	E	AC**U**	AC**C**	–
1577	E	G**C**G	G**A**G	Ala→Glu
1819	E	**A**GG	**C**GG	Lys→Arg
3051	NS1	CU**G**	CU**A**	–
3332	NS1	G**G**A	G**A**A	Gly→Glu
3553	NS2A	**C**UG	**A**UG	Leu→Met
3609	NS2A	AU**U**	AU**C**	–
4328	NS2B	G**U**G	G**C**G	Val→Ala
4437	NS2B	GU**U**	GU**G**	–
5793	NS3	UG**C**	UG**U**	–
5805	NS3	GA**C**	GA**U**	–
6509	NS4A	G**G**G	G**U**G	Gly→Val
6620	NS4A	G**C**U	G**U**U	Ala→Val
6681	NS4A	AA**A**	AA**G**	–
6709	NS4A	**A**UA	**G**UA	Ile→Val
6846	NS4A	GA**C**	GA**U**	–
943	NS4B	**A**GC	**G**GC	Ser→Gly
6954	NS4B	UU**A**	UU**G**	–
6962	NS4B	AA**G**	A**G**G	Lys→Arg
7205	NS4B	G**C**C	G**U**C	Ala→Val
7218	NS4B	GG**C**	GG**U**	–
8290	NS5	**G**UA	**A**UA	Val→Ile
9511	NS5	**C**CC	**A**CC	Pro→Thr
10391	3′-UTR	**76 nt insert**	–	–
10399	3′-UTR	**C**	**U**	–
10763	3′-UTR	**U**	**A**	–
10929	3′-UTR	**U**	**C**	–
10948	3′-UTR	**G**	**U**	–

[a] Coordinates according to the 956D117B3 sequence (GenBank #M12294).

Phenotypic differences between parent B956, isolate 956D117B3 and viral molecular clone WN1415

956D117B3 and WN1415 display a substantially decreased cytopathicity as compared to the B956 strain. In contrast to the latter, both do not form distinguishable foci of cytopathology (plaques) in Vero cells (Fig. 1 for WN1415 and B956). However, foci of virus multiplication are easily detectable by immunostaining with the DAB-peroxidase procedure (Fig. 1). Despite the low cytopathicity, both WN1415 and 956D117B3 grow in Vero cells to high titers, which in optimal conditions may reach 10^8–10^9 pfu/ml. The more cytopathic B956 strain produced titers in the range of 10^7 pfu/ml. There was no difference between viruses in plaque formation in BHK cells.

Peripheral virulence was assessed in 4-week-old outbred mice, which were infected i.m. with 10-fold virus dilutions. The control group of 6 mice received the diluent only. As shown in Table 2, 100% mortality could not be achieved even at highest i.m. dose used. LD_{50} for WN1415 by this route was found to be 6.6×10^6 pfu. The 956D117B3 virus, which was not molecularly cloned, showed a slightly higher virulence with LD_{50} of 1.1×10^6 pfu for i.m. inoculation (Table 2). By the i.c. route, WN1415 showed LD_{50} of 158 pfu for adult 7-week-old mice, but the uncloned 956D117B3 isolate appeared about 10-fold more neurovirulent with LD_{50} of only 13.5 pfu (Table 3). The observed differences may have resulted from the presence of quasi-species with a higher virulence in the uncloned 956D117B3 population. There was no heterogeneity in size of 956D117B3 multiplication foci both in Vero and in BHK cells, which is consistent with the fact that the RNA from which the virus was rescued had been extracted from a triple plaque purified virus (Wengler and Beato, 1979). As mentioned above, consensus sequencing did reveal several heterogeneous loci in the 956D117B3 genome indicative of the presence of quasi-species, although we did not perform detailed functional analysis of these. The parent B956 strain was found to be about 50 times more virulent than WN1415 upon i.m. infection, with LD_{50} of 1.24×10^5 pfu (Table 4). In a sharp contrast to the B956 group, LD_{50} for NY99 was found to be only 19 pfu (Table 4), which agrees with earlier reports (Beasley et al., 2002) documenting the high pathogenicity of this strain. Similar results were obtained after i.p. inoculation of NY99 as well (data not shown).

Infection with lethal doses of NY99 uniformly resulted in rapidly progressing encephalitis accompanied by para- or even quadriplegia and resolved by death often in less than 12 h after onset of neurological symptoms. In contrast, infection with lethal doses of WN1415 resolved in 2–3 days and was characterized by immobility, anorexia, and a substantial weight loss, but paralytic symptoms were uncommon. Neurological symptoms such as paralysis more often were observed with 956D117B3, thus corroborating its heterogeneous nature. The provided evidence indicates that clonal purification of 956D117B3 using the infectious clone technology has yielded the homogeneous viral clone characterized by a lower residual virulence in the mouse model.

Virus-specific humoral response

For both 956D117B3 and its WN1415 clonal isolate, the identity with the NY99 strain is only 79.4% at the nucleotide level, thus confirming assignment of two strains to separate lineages (Berthet et al., 1997; Lanciotti et al., 1999). The overall identity of both viruses at the level of the amino acid sequence is 93.8%, which is unevenly distributed among individual proteins (Table 5). At the 94% sequence identity between envelope proteins of NY99 and WN1415, induction of cross-reactive antibodies and a certain level of cross-protective immunity could be expected. To evaluate that, titers of NY99 cross-reacting antibodies and the neutralizing activity of the immune sera

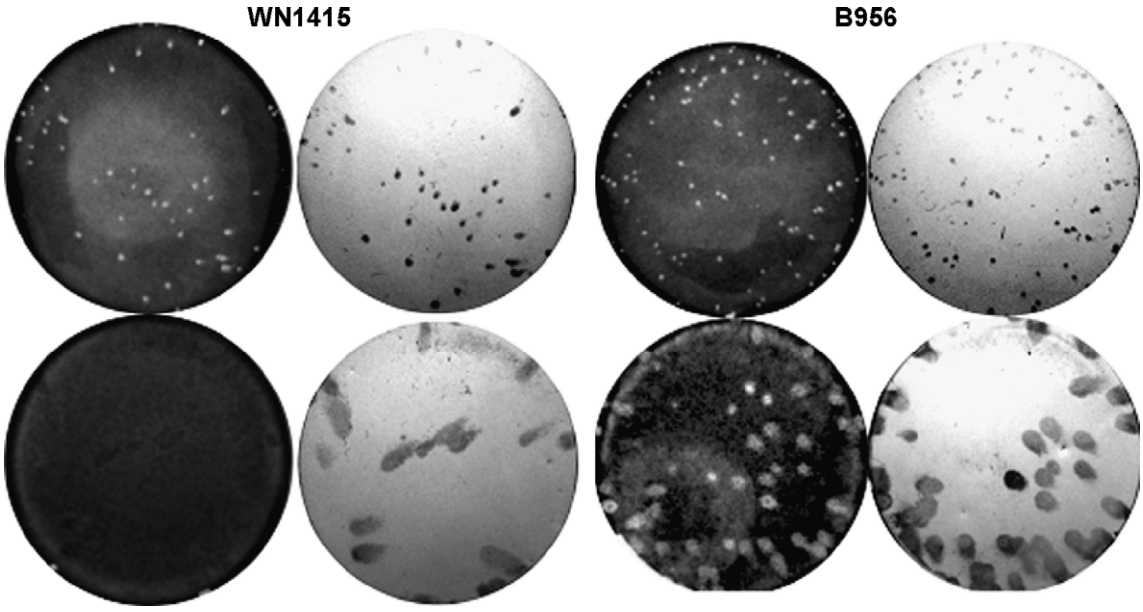

Fig. 1. Cytopathic properties of the WN prototype strain B956 and the clonal isolate WN1415. BHK (top panel) or Vero (lower panel) monolayers were infected with 10-fold dilutions of corresponding viruses and incubated under 1% methylcellulose overlays for 4–5 days. Cells fixed with 1% HCHO were stained with Coumassie G250, washed, dried and photographed in visible light (left panels for each virus). Coumassie stain was removed with ethanol washes, and monolayers were rehydrated in 50% alcohol followed by PBS. Virus foci were immunostained as described in Materials and methods and photographed in visible light (right panels for each virus).

against the NY99 strain were determined in mice, which survived immunization with 956D117B3 or WN1415. Both 956D117B3 and NY99 antigens produced very similar readings with the tested sera in ELISAs specific for antiviral IgG. The levels of WN-specific IgG at 3 weeks post-immunization with WN1415 and at 2 weeks post-challenge with NY99 are shown in Fig. 2 as endpoint dilutions of averaged sera yielding positive readouts with the NY99 antigen. Higher virus doses resulted in earlier appearance of WN-specific antibodies (data not shown), and all survived mice developed detectable antiviral humoral immunity by 3 weeks post-infection, in serum dilutions ranging from 400 to 6400 depending on virus dosage. By this time, mice in all dosage groups have already demonstrated substantial titers of neutralizing antibodies against the NY99 strain (Table 6). Thus, combination of virus IgG-specific ELISA and data on neutralizing antibody titers confirmed the high antigenic similarity of both viruses, and suggest that efficient protection against NY99 infection may be expected after WN1415 immunization as well.

Table 2
Virulence of WN1415 and 956D117B3 in NIH Swiss mice by the i.m. route

WN1415[a]	D/T[b]	%[b]	956D117B3[a]	D/T[b]	%[b]
4.9E + 07	5/6	83	4.0E + 07	4/6	66
4.9E + 06	2/6	33	4.0E + 06	4/6	66
4.9E + 05	1/6	17	4.0E + 05	3/6	50
4.9E + 04	1/6	17	4.0E + 04	1/6	17
4.9E + 03	0/6	0	4.0E + 03	0/6	0
4.9E + 02	0/6	0	4.0E + 02	0/6	0
0	0/6	0	0	0/6	0
LD_{50} = 6.62E + 06			LD_{50} = 1.13E + 06		

[a] pfu per mouse.
[b] Mortality after 2 weeks (D/T—dead/total).

Immunization with WN1415 confers strong protection against NY99 challenge

Three weeks after inoculation with various doses of WN1415 (as shown in Table 7), all survived mice were challenged i.m. with 10 LD_{50} of NY99 strain. Eighty-five percent of mice, which had survived the WN1415 infection, have survived the lethal challenge with NY99, whereas none survived in the control group (Table 7). Although 100% protection was observed only in mice that received the two highest doses of WN1415, 67% of mice immunized with only 55 pfu of WN1415 where able to fend off the infection. During the 2-week observation period after the challenge, neither of mice that survived the challenge have displayed any signs of disease, such as immobility, ruffled fur, or anorexia, while all mice in the control group have succumbed to paralytic encephalitis between 8 and 11 days

Table 3
Infectivity of WN1415 and 956D117B3 in NIH Swiss mice by the i.c. route

WN1415[a]	D/T[b]	%[b]	956D117B[a]	D/T[b]	%[b]
–	–	–	5.2E + 03	3/3	100
5E + 02	3/3	100	5.2E + 02	6/6	100
5E + 01	0/3	0	5.2E + 01	5/6	83
5E + 00	0/3	0	5.2E + 00	1/3	33
5E−01	0/3	0	–	–	–
LD_{50} = 1.58E + 02			LD_{50} = 1.35E + 01		

[a] pfu per mouse.
[b] Mortality after 2 weeks (D/T—dead/total).

Table 4
Virulence of B956 and NY99 in NIH Swiss mice by the i.m. route

B956[a]	D/T[b]	%[b]	NY99[a]	D/T[b]	%[b]
2.2E + 06	5/6	83	–	–	–
2.2E + 05	4/6	66	1E + 05	6/6	100
2.2E + 04	1/6	17	1E + 04	6/6	100
2.2E + 03	0/6	0	1E + 03	5/6	83
2.2E + 02	0/6	0	1E + 02	5/6	83
2.2E + 01	0/6	0	1E + 01	3/6	50
0	0/6	0	0	0/6	0
LD_{50} = 1.24E + 05			LD_{50} = 1.93E + 01		

[a] pfu per mouse.
[b] Mortality after 2 weeks (D/T—dead/total).

post-challenge. In the three highest WN1415 dosage groups, mice did not display statistically significant changes in the titer of virus specific antibodies after the challenge (Fig. 2), indicating that infection with NY99 was very limited or was completely suppressed. In contrast, mice that survived the challenge in the two lowest WN1415 dosage groups did show a significant increase in average antibody titers by 2 weeks after challenge (Fig. 2, P values 0.026 and 0.022 for 5.5E + 02 and 5.5E + 01 pfu correspondingly), indicating that infection with NY99 virus was not prevented. However, we have not observed any disease symptoms in these mice. Whether immunization with lower doses of WN1415 has induced a potent cell-mediated immunity, which has helped to curb and eventually eliminate infection, requires a more detailed investigation.

Inoculation with the more virulent 956D117B3 isolate resulted in a higher mortality rate (Table 2), but has also conferred a stronger protection upon survived mice since 100% survival was observed in all dosage groups after i.p. challenge with 10-fold higher doses of NY99 (100 i.p. LD_{50}; Table 7). While older mice have been used in this experiment, the age did not appear to contribute to the observed increase in survival since the challenge with NY99 still resulted in 100% mortality in the control group of mice (Table 7). These data suggest that more virulent quasi-species present in the 956D117B3 population may have substantially increased the potency of the preparation. On the other hand, while less virulent WN1415 has obviously lost some potency, it was still able to induce a sufficient protection in immunized animals.

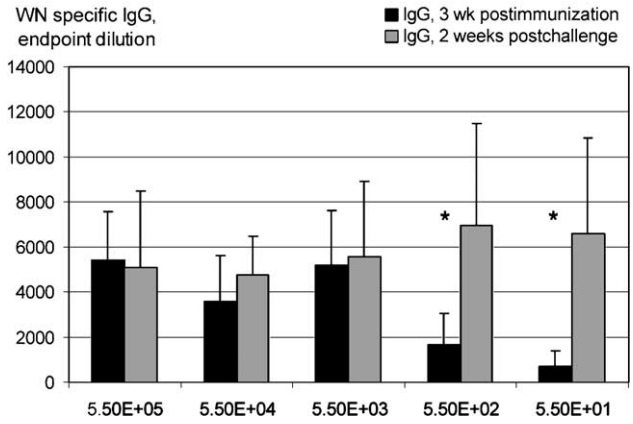

Fig. 2. Development of WN-specific IgG response. Four weeks old NIH Swiss mice were immunized i.m. with indicated doses of WN1415 and sera collected at 3 weeks post-infection and at 2 weeks post-challenge with 10 LD_{50} of WN NY99. Serum samples were assayed by ELISA for WN-specific IgG using the NY99 viral antigen and endpoint dilution values were averaged for survivors in each of the WN1415 dosage groups. Pre- and post-challenge IgG titers were compared using standard T-test. In the three highest WN1415 dosage groups, mice did not display statistically significant changes in the titer of virus specific antibodies after the challenge ($p > 0.05$), as compared to mice in the two lowest dosage group ($P < 0.05$; see text for details).

Discussion

Despite tremendous advances in our understanding of viral replication and pathogenesis, the attenuation process as a methodology of vaccine design continues to rely on a poorly rationalized and largely empirical approach. Beyond YF17D, only a few more attenuated flavivirus vaccines have been licensed for human use or have reached the level of human clinical trials elsewhere in the world (reviewed, (Pugachev et al., 2003)). The rational approach has been more successful in design of chimeric or subunit vaccines (Eckels and Putnak, 2003; Lai and Monath, 2003), which despite efficient stimulation of humoral immunity remains much less efficient by design in induction of virus-specific cell-mediated immune responses. For this reason, development of the most potent attenuated virus vaccines remains an important and viable alternative.

In this report, we described the WN1415 molecular viral clone that is a descendant of the West Nile prototype strain B956. WN1415 was produced from 956D117B3 isolate of B956 virus using molecular infectious clone technology. 956D117B3 has undergone numerous passages and differs from the parent strain both by genotype and phenotype, including the lower cytopathicity in cell culture and a reduced virulence in mice. The majority of amino acid substitutions were found in nonstructural proteins and in the 3′-untranslated region suggesting changes at the intracellular stage of viral replication. However, the non-conservative change Ala→Glu at pos. E_{204} might also contribute to attenuation.

Table 5
The identity of WN strains NY99[a] and WN1415[b] at the protein level

	C	prM(M)	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5
% identity	97	96 (96)	94	92	89	98	96	93	91	95

[a] GenBank #AF196835.
[b] GenBank #M12294.

Table 6
Development of NY99 neutralizing immunity[a] after WN1415 immunization[b]

Mouse No.	WN1415 dose, pfu/mouse				
	5.5E + 05	5.5E + 04	5.5E + 03	5.5E + 02	5.5E + 01
1	160	ND	320	160	ND
2	80	320	80	80	ND
3	ND	160	160	40	160
4	160	80	ND	ND	160
5	160	ND	ND	80	ND
6	40	320	640	160	40

[a] NY99 neutralizing titer at 3 weeks post-immunization.
[b] ND—mice died after either the primary immunization, or the challenge, or for unrelated reasons.

This position is located to the loop between strands f and g in the dimerization domain (Rey et al., 1995), and both more virulent B956 and NY99, as well as all JE viruses sequenced to date contain an uncharged amino acid at this position. On the other hand, attenuation does not always result in accumulation of mutations in the E protein (Kinney et al., 1997), and several non-conservative changes found in NS1, NS4B and NS5, as well as the 76 nt deletion in 3′-NTR may contribute to attenuation at the level of RNA replication or other stages of the virus infectious cycle.

Lineage 2 WN viruses have not been associated with epidemic or epizootic outbreaks (Lanciotti et al., 1999), although a few virulent isolates have been identified at the WHO Collaborating Center for Tropical Diseases (Beasley et al., 2002) in collection of WN strains isolated between 1950 and 1999 from various hosts. The B956 strain itself is 6,500-fold less virulent as compared to NY99 strain (LD_{50} are 1.24×10^5 and 19 pfu for B956 and WN NY99, respectively, for peripheral inoculation) and its additional 50-fold attenuation makes WN1415 ($LD_{50} = 6.62 \times 10^6$) an attractive candidate for development of an attenuated WN vaccine. Similar to the YF17D vaccine, which showed i.c. LD_{50} of 0.3 to 5.9 pfu depending on manufacturer (calculated based on Monath, 1999), the WN1415 isolate remains neurovirulent for adult mice after i.c. inoculation.

It was not clear whether a lineage 2 virus is capable to induce a strong protective immunity against the highly pathogenic lineage 1 NY99 strain and will not result in an opposite effect of the immune enhancement, which had been described for homologous and heterologous viruses of the JE serocomplex and WN in particular both in mice (Fagbami et al., 1987; Gollins and Porterfield, 1985; Lobigs et al., 2003) and observed with sera of naturally immunized human population (Fagbami et al., 1988). The sequence identity between E proteins of two viruses is 94% corresponding to 30 aa differences (including a 4-aa deletion encompassing the only glycosylation site in the E protein) out of 501 amino acids composing the E protein of NY99. Recently reported variation in the neutralizing capacity toward another lineage 2 WN strain H-442 of three NY99 monoclonal antibodies (MAbs) corroborate antigenic differences between the two lineages (Beasley and Barrett, 2002). Interestingly, however, that for the tested MAbs, two out of three experimentally determined epitope-defining positions (E_{307} and E_{330}, (Beasley and Barrett, 2002)) appeared identical in both tested strains (GenBank #AF459403 and #AF196835), indicating that analyzed neutralizing epitopes were not linear and the linear homology has a limited predictive value in estimation of the antigenic similarity.

The present evidence indicates that immunization with attenuated derivatives of B956, the 956D117B3 isolate and its clonal variant WN1415, induces potent immune responses capable of protecting against infection with the highly virulent NY99 strain. Immunization with doses in the range of 5.5×10^3 to 5.5×10^5 pfu of WN1415 (roughly equivalent to 35–3500 mouse i.c. LD_{50}) induced a substantial level of NY99 neutralizing antibodies detectable as early as 3 weeks post-immunization and the resulting immunity protected almost all immunized animals from infection with NY99. Although a direct comparison cannot be made, a single human dose of YF17D vaccine is required to contain at least 1000 mouse i.c. LD_{50} (WHO, 1998), but in fact, most preparations contain between 5000 and 10,000 LD_{50} per dose (Barrett, 1997). The majority of mice immunized with doses 55–550 pfu (0.35–3.5 mouse i.c. LD_{50}) also withstood the challenge, even though the infection was not prevented as indicated by the several fold increase in post-challenge IgG titers. Since cell-mediated immunity plays a major role in clearance of viral infection (Engle and Diamond, 2003; Murphy and Chanock, 1996), one may suggest that immunization with lower doses of WN1415 induced a cell-mediated response strong enough to terminate developing infection. Studies on the status of cell-mediated immunity after immunization with WN1415 are currently under way.

The more virulent 956D117B3 isolate has shown a higher potency in our study, protecting all mice even in the group immunized with a dose of 10 pfu. Perhaps, due to its higher potency, the 956D117B3 isolate may seem a better candidate for further development of a WN vaccine. However, its unknown passage history is a major stumbling block for its use in vaccine development. In contrast, the WN1415 virus can be recovered again from infectious RNA and further propagated using approved substrates to create a defined passage history. We have used the molecular cloning technology to improve homogeneity of the viral

Table 7
Survival of immunized mice after NY99 challenge

WN1415[a]	S1/S2[b]	%[b]	956D117B3[a]	S1/S2[b]	%[b]
5.5E + 05	5/5	100	1E + 06	2/2	100
5.5E + 04	4/4	100	1E + 05	4/4	100
5.5E + 03	5/4	80	1E + 04	4/4	100
5.5E + 02	6/5	83	1E + 03	5/5	100
5.5E + 01	6/4	67	1E + 02	5/5	100
0	6/0	0	0	6/0	0
NY99 dose: 10 LD_{50}, i.m.			NY99 dose: 100 LD_{50}, i.p.		

[a] Primary immunization, pfu/mouse.
[b] S1—survived after primary immunization; S2—survived the challenge.

population and eliminate adventitious agents, which could be carried over its passage history. The pWN1415 infectious clone was reconstructed from molecularly cloned cDNA fragments, and the virus with the predominant genotype was rescued from synthetic RNA (Yamshchikov et al., 2001). The obtained more homogenous viral population displayed a decreased virulence in the mouse model, which is not necessarily a trivial result considering that an earlier attempt to apply the molecular clone technology to improve the safety profile of the YF17D vaccine had yielded a more virulent isolate (Marchevsky et al., 1995).

Despite already achieved the relatively high level of attenuation, the WN1415 virus still causes limited mortality of infected animals when used at high doses. The existing attenuated vaccines approved for human use, JE SA14-14-2 and YF17D, do not cause mortality upon peripheral inoculation of adult mice. WN and JE viruses belong to one serological group, and genetic basis of SA14-14-2 attenuation is well established (Aihara et al., 1991; Arroyo et al., 2001a; Monath et al., 2002; Ni and Barrett, 1996; Ni et al., 1995; Wu and Lee, 2001). It was noted that virulent JE and WN strains contain identical amino acids in E protein loci that are crucial for JE attenuation (Arroyo et al., 2001b; Monath et al., 2001, 2002). This defines a feasible approach for further attenuation of the WN1415 vaccine candidate.

While attenuation of the NY99 virus or its closer lineage 1 homologue presumably could provide a better match for development of a WN vaccine, this approach is immensely complicated by the very high and age-independent virulence and pathogenicity of this strain (Beasley et al., 2002). Several strains of Kunjin virus, an Australian antigenic relative of lineage 1 WN viruses (Scherret et al., 2001), have displayed a reduced virulence in the mouse model (Beasley et al., 2002), but their protective potential against NY99 infection has yet to be evaluated. The efficient protection induced after immunization with the attenuated molecular clone WN1415, strengthened by the availability of its highly stable infectious clone, and combined with the current knowledge of flavivirus attenuation may bring the goal of WN vaccine design within a practically reachable limits.

Materials and methods

Cells and viruses

BHK-21 cells (Bredenbeek et al., 1993) and Vero (ATCC CRL-1586) were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS; Hyclone, Logan, UT) and 1× antibiotic–antimycotic mixture (Invitrogen, Carlsbad, CA). WN strain 956D117B3 was recovered after transfection of BHK cells with archival RNA (Wengler and Beato, 1979; Yamshchikov et al., 2001) and was used after an additional passage in Vero cells. Isolate WN1415 used in this study was recovered after transfection of synthetic RNA prepared from the pSP6WN/Xba infectious clone (Yamshchikov et al., 2001) into BHK cells and was used at passage 2 in Vero cells. The prototype WN strain B956 (at mouse brain passage 2) and a 385–99 isolate of the NY99 strain (at Vero passage 1) were generously provided by Drs. R. Shope and R. Tesh (Galveston, TX), respectively. The viruses were grown once in C6/36 cells and were used after an additional passage in Vero and C6/36 cells, respectively.

Nucleotide sequence determination

Consensus sequencing (Pugachev et al., 2002) was done after RT-PCR amplification of eight overlapping cDNA fragments spanning the entire genome of WN1415, 956D117B3 or B956 viruses. The gel-purified fragments were sequenced without intermediate subcloning steps using the ABI310 Genetic Analyzer (ABI, Foster City, CA). A set of sequencing primers was designed to provide overlapping sequence segments covering the entire genome in both directions. Contiguous sequences were assembled with AutoAssembler 2.0 software (ABI) and downstream sequence analysis was done with the VectorNTI Advance suite (InforMax, Bethesda, MD). Folding of 3′NTR segment of genomic RNA was done with the MFOLD program available on-line (Zuker, 2003). Sequence of the prototype B956 strain is deposited in the GenBank under accession #AY532665.

Virus infection and challenge

Swiss Webster (ICR) or NIH Swiss outbred mice were purchased from Hilltop Lab Animals Inc. (Scottsdale, PA) and Harlan Sprague–Dawley (Indianapolis, IN), respectively, maintained in a BL3 facility according to the NIH guidelines, and used in IACUC-approved protocols. Mice were anesthetized with metofane (Mallinckrodt Veterinary, Mundelein, IL) and infected into the *Tibialis anterior* muscle (i.m.) or intracerebrally (i.c.) with virus diluted in PBS plus 0.2% of normal mouse serum. LD_{50} (50% lethal dose) was calculated by the Reed and Muench method (Burleson et al., 1992). For challenge experiments, anesthetized mice were inoculated i.m. with 10 i.m. LD_{50} (unless specified otherwise) of NY99 virus. Mice were observed daily for the development of encephalitis and mortality. Moribund mice were euthanased.

Analysis of antibody response

For ELISA, the coating antigen was prepared from 956D117B3 or NY99 viruses pelleted through 20% sucrose cushion from media of infected Vero cells. The 3-h centrifugation at 25,000 rpm was done using a Beckman SW28 rotor. The pellet was resuspended in 50 mM triethanolamine–HCl pH 8.5, 100 mM NaCl, and

0.5% Triton X-100 to extract viral envelope subunit antigen (Heinz et al., 1981). Clarified supernatant (1000 × *g*, 15 min, 4 °C) was stored at −80 °C and was used at 1:500 dilution in PBS. Serial 2-fold dilutions of immune sera were tested in duplicate using a goat anti-mouse IgG(H + L) peroxidase conjugate with the TMB substrate (Sigma-Aldrich, St. Louis, MO). Assays were read at 450 nm using PowerWave XS microplate reader equipped with KC-4 software (Bio-Tek, Winooski, VT). Readings of two standard deviations above the background (serum from control mice used at the same dilution) were considered positive.

Virus titration and plaque reduction-neutralization (PRNT) assays

Virus titers were determined by a microplate method. Serial 10-fold dilutions of virus stocks in DMEM containing 0.5% FCS were prepared in duplicate wells of a 96-well cluster and 50 μl was transferred in parallel to confluent monolayers of Vero cells in 96-well plates using a multi-channel pipettor. The plates were incubated for 1 h at 37 °C in the CO_2 incubator with occasional shaking. The inocula were aspirated, replaced with 100 μl of the growth medium, and plates returned to the incubator for additional 24 h. Under these conditions, virus multiplication foci consisted of compact clusters of 5–15 cells, which stained positive for the viral antigen as described below.

PRNT assays were done in a similar microplate format. Two-fold dilutions of immune sera in DMEM + 0.5% FCS were mixed in duplicate in 96 well plates with equal volume of NY99 virus prediluted in the same medium to 2×10^3 pfu/ml and the plate was kept in CO_2 incubator at 37 °C for 1 h. Fifty microliters of each mix (containing about 50 pfu) was transferred in parallel into a 96-well plate with confluent Vero monolayers and incubated for another hour as above. The inocula were aspirated, replaced with 100 μl of the growth medium and plates returned to the incubator for 24 h.

At the end of the incubation period, cells were fixed by addition of 25 μl/well of 10% formalin in PBS and incubation for 30 min at room temperature. Foci of viral multiplication were visualized on fixed monolayers with DAB substrate (Vector Laboratories, Burlingame, CA) after the following treatment sequence (50 μl/well): 0.5% Thesit (Sigma-Aldrich) for 10 min, 1:1000 dilution of WN mouse hyperimmune antiserum for 30 min, 1:1000 dilution of biotinylated horse anti-mouse IgG(H + L) (Vector Laboratories) for 30 min, 2 μg/ml streptavidin (ICN, Aurora, OH) for 30 min, 3.5 μg/ml biotinylated peroxidase (ICN) for 30 min. PBS supplemented with 1% horse serum was used as a diluent throughout the assay, and plates were washed extensively in tap water between treatments. For PRNT, endpoint serum dilutions providing 50% reduction in the number of foci over control wells that contained no immune serum were counted as positive.

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